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The *Cladosporium fulvum* Virulence Protein Avr2 Inhibits Host Proteases Required for Basal Defense [W][OA]

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Cladosporium fulvum* (syn. *Passalora fulva*) is a biotrophic fungal pathogen that causes leaf mold of tomato (*Solanum lycopersicum*). During growth in the apoplast, the fungus establishes disease by secreting effector proteins, 10 of which have been characterized. We have previously shown that the Avr2 effector interacts with the apoplastic tomato Cys protease Rcr3, which is required for Cf-2–mediated immunity. We now show that Avr2 is a genuine virulence factor of *C. fulvum*. Heterologous expression of *Avr2* in *Arabidopsis thaliana* causes enhanced susceptibility toward extracellular fungal pathogens, including *Botrytis cinerea* and *Verticillium dahliae*, and microarray analysis showed that *Avr2* expression triggers a global transcriptome reflecting pathogen challenge. Cys protease activity profiling showed that Avr2 inhibits multiple extracellular *Arabidopsis* Cys proteases. In tomato, *Avr2* expression caused enhanced susceptibility toward Avr2-defective *C. fulvum* strains and also toward *B. cinerea* and *V. dahliae*. Cys protease activity profiling in tomato revealed that, in this plant also, Avr2 inhibits multiple extracellular Cys proteases, including Rcr3 and its close relative Pip1. Finally, silencing of *Avr2* significantly compromised *C. fulvum* virulence on tomato. We conclude that Avr2 is a genuine virulence factor of *C. fulvum* that inhibits several Cys proteases required for plant basal defense.

INTRODUCTION

Cladosporium fulvum (syn. *Passalora fulva*) is a biotrophic fungal pathogen that causes leaf mold of tomato (*Solanum lycopersicum*) (Joosten and de Wit, 1999; Thomma et al., 2005). Similar to other plant pathogenic Mycosphaerellaceae, host colonization is characterized by strict extracellular growth in the apoplastic space surrounding the leaf mesophyll without formation of haustoria (Bond, 1938; Lazarovits and Higgins, 1976; de Wit, 1977; Thomma et al., 2005). Ten *C. fulvum* effector proteins secreted during host colonization have been characterized, the highest number for any filamentous plant pathogen studied so far (Thomma et al., 2005). Four of these effectors are race-specific avirulence proteins (Avr2, Avr4, Avr4E, and Avr9), six are extracellular proteins (Ecp1, Ecp2, Ecp4, Ecp5, Ecp6, and Ecp7), and all of the corresponding genes have been cloned (van Kan et al., 1991; van den Ackerveken et al., 1993; Joosten et al., 1994; Laugé et al., 2000; Luderer et al., 2002; Westerink et al., 2004; Bolton et al., 2008). Race-specific resistance against *C. fulvum* in tomato is governed in a gene-for-gene manner by dominant *C. fulvum* (*Cf*) resistance genes that mediate activation of a defense cascade, culminating in a hypersensitive response (HR) and host immunity (Joosten and de Wit, 1999; Rivas and Thomas, 2005). It has recently been demonstrated that the *C. fulvum* effector protein Avr4 (Joosten et al., 1994) contributes to full virulence by protecting fungal hyphae against hydrolysis by plant chitinases (van den Burg et al., 2006; van Esse et al., 2007). In addition, a putative intrinsic function was assigned to the Avr2 effector. In the incompatible interaction, Avr2 was shown to physically interact with, and inhibit, the extracellular papain-like Cys protease Rcr3 (required for *C. fulvum* resistance; Krüger et al., 2002), which, in resistant tomato varieties, is guarded by the extracellular membrane-anchored resistance protein Cf-2 (Rooney et al., 2005). Interestingly, the Rcr3^{esc} variant that occurs in *S. esculentum* is a functional Cys protease with a mutation outside the active center of the enzyme that causes chronic necrosis in mature tomato plants carrying *Cf-2* (Krüger et al., 2002; Rooney et al., 2005). Thus, binding of Avr2 results in a conformational change of Rcr3 that is monitored by the Cf-2 protein, resulting in HR and resistance against *C. fulvum* isolates that produce wild-type Avr2 (Rooney et al., 2005).

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Pathogens and their hosts use both proteases and protease inhibitors during their interactions to combat each other (van der Hoorn, 2008). Based on catalytic activity, the proteases are classified as Asp, Cys, metallo-, Ser, and Thr proteases (Rawlings et al., 2006). These main classes have been further subdivided into clans and families based on evolutionary relationships. In the *Arabidopsis thaliana* genome, >800 protease genes have been identified, of which ~140 are Cys proteases, including ~40 papain-like proteases (van der Hoorn, 2008). Several Cys proteases have been demonstrated to play a role in programmed cell death–like responses, including pathogen-triggered HR (Beers et al., 2000; Rojo et al., 2004; Suarez et al., 2004; Bozhkov et al., 2005; Kuroyanagi et al., 2005; Rooney et al., 2005; Hatsugai et al., 2006; Gilroy et al., 2007; Mur et al., 2007). Similar to Avr2, a number of secreted effector proteins with protease inhibitory activity in tomato have been identified from the oomycete pathogen *Phytophthora infestans* (Tian et al., 2004, 2005, 2007). For example, a Kazal-like Ser protease inhibitor targets the extracellular subtilisin-like protease P69B (Tian et al., 2004), and the Cys protease inhibitor EPIC2, although structurally unrelated to Avr2, targets the Rcr3-like Cys protease Pip1 (*Phytophthora*-inhibited protease; Tian et al., 2007). It is tempting to speculate that protease inhibitors, such as *C. fulvum* Avr2 and *P. infestans* EPIC2, inactivate basal host defense by suppressing host protease activity, but so far it has not been demonstrated that these protease inhibitors genuinely contribute to pathogen virulence or that the targeted plant proteases are required for basal host defense.

In this study, we show that expression of *C. fulvum Avr2* in both *Arabidopsis* and tomato enhances susceptibility toward a number of fungal pathogens, including race 2 strains of *C. fulvum* that lack functional Avr2. Transcriptome analysis in *Arabidopsis* was employed to demonstrate that Avr2 does not merely disrupt normal host physiology, but triggers a global transcriptional reprogramming that reflects a typical host response to pathogen attack. Protease activity profiling was used to identify multiple host proteases in *Arabidopsis* and tomato that are inhibited by Avr2. Finally, we demonstrate with RNA interference–mediated gene silencing that Avr2 contributes to *C. fulvum* virulence. Overall, the results of this study demonstrate that Avr2 is a genuine virulence factor of *C. fulvum* that inhibits a set of Cys proteases that may be essential for basal host defense.

RESULTS

Heterologous Expression of *C. fulvum Avr2* in *Arabidopsis* Enhances Susceptibility toward Distinct Fungal Pathogens

Basal defense against microbial pathogens is well characterized in the model plant *Arabidopsis* (Thomma et al., 2001). To assess whether any of the *C. fulvum* effector proteins targets conserved basal defense responses, transgenic *Arabidopsis* lines in wild-type Columbia (Col-0) were generated that constitutively produce individual *C. fulvum* effector proteins. After segregation analyses, several independent homozygous single-integration lines were obtained for the *C. fulvum* effector genes *Avr2*, *Avr4*, *Avr4E*, *Avr9*, *Ecp2*, *Ecp4*, or *Ecp5*. In a screen for attenuated basal defense, for each individual effector molecule three randomly chosen lines were challenged with the fungal pathogen *Botrytis cinerea* (Thomma et al., 1998, 1999a). In addition to increased susceptibility in the lines that express *Avr4* (van Esse et al., 2007), lines expressing *Avr2* also showed significantly enhanced gray mold disease, while transformants expressing any of the remaining effectors did not show clear alterations in disease susceptibility. As a result, the *Avr2*-expressing *Arabidopsis* lines were subjected to further analysis.

To select lines with the highest level of Avr2 production, total protein extracts were screened in protein gel blot analyses using Avr2-specific polyclonal antibodies (van Esse et al., 2006). Three lines with the highest levels of Avr2 production were retained for further analyses (*At-Avr2-A* to *-C*, collectively called *At-Avr2* lines). When grown under standard greenhouse or climate chamber conditions, the *At-Avr2* lines did not show macroscopically visible phenotypic anomalies (see Supplemental Figures 1A and 1B online). To confirm the presence of biologically active Avr2 in the apoplast of these transgenic lines, apoplastic fluid from *At-Avr2* lines was injected into leaves of a tomato *Cf-2* plant that resulted in a typical HR, while injection of apoplastic fluid from progenitor wild-type Col-0 plants showed only slight nonspecific chlorosis (see Supplemental Figure 1C online). Subsequently, the *At-Avr2* lines were challenged with the *Arabidopsis* fungal pathogens *Alternaria brassicicola*, *B. cinerea*, and *Plectosphaerella cucumerina*, the oomycete pathogens *Hyaloperonospora parasitica* and *Phytophthora brassicae*, and the bacterium *Pseudomonas syringae* pv *tomato* strain DC3000, using both progenitor Col-0 plants and *Avr9*-expressing *Arabidopsis* plants as controls. In these assays, *At-Avr2* lines inoculated with the necrotrophic fungal pathogens *B. cinerea* or *P. cucumerina* showed a clear enhancement of disease progression when compared with the inoculated control plants (Figure 1).

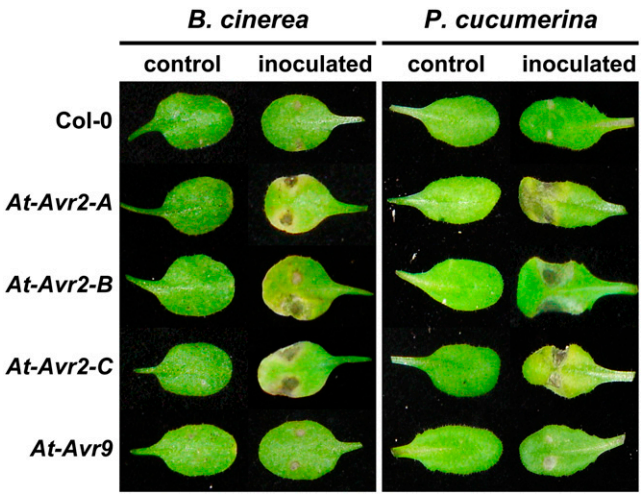

Figure 1. *Avr2*-Expressing *Arabidopsis* Is More Susceptible to the Fungal Pathogens *B. cinerea* and *P. cucumerina*.

Typical symptoms caused by *B. cinerea* and *P. cucumerina* on 4-week-old plants of three independent *Avr2*-expressing *Arabidopsis* lines (*At-Avr2-A* to *-C*) at 4 DAI. Typical symptoms on the progenitor Col-0 line and an *Avr9*-expressing transgenic line (*At-Avr9*) are shown as controls.

On the *At-Avr2* lines, necrotic lesions developed faster and grew larger compared with inoculated control plants on which lesions remained small (Figure 1; see Supplemental Figure 2 online). Generally, disease progression did not result in sporulation of the pathogens on *At-Avr2* lines, although occasionally sporulation of *P. cucumerina* was observed on some of the inoculated leaves. Quantitative degrees of resistance are typical for defense against necrotrophic pathogens (Thomma et al., 1998, 1999a, 1999b; Kliebenstein et al., 2005). Interestingly, inoculation with another necrotrophic fungus, *A. brassicicola*, did not result in a more susceptible phenotype, indicating that the enhanced susceptibility is pathogen dependent and not due to an overactive host defense. Likewise, inoculation with the bacterial pathogen *P. syringae* pv *tomato* strain DC3000, or the oomycete pathogens *P. brassicae* and *H. parasitica* strains Waco9 (virulent) and Cala2 (avirulent), did not reveal increased susceptibility of *At-Avr2* lines. It has been shown previously that basal defense responses show differential effectivity toward different microbial pathogens (Thomma et al., 1998; 1999a, 1999b, 2000, 2001). Taken together, expression and extracellular targeting of Avr2 in *Arabidopsis* promotes the virulence of some, but not all, pathogens.

Avr2 Expression in *Arabidopsis* Leads to Transcriptional Reprogramming Reflecting Defense Responses after Pathogen Challenge

To investigate whether *Avr2* expression merely disturbs host physiology, or specifically interferes with basal host defenses, transcriptional profiling in the absence of pathogen challenge was performed on 4-week-old *At-Avr2-A* plants and progenitor Col-0 plants using Affymetrix ATH1 whole-genome arrays. In total, 880 genes were found to be significantly regulated (Bayesian *t* testing, $P < 0.05$) in response to *Avr2* expression. To gain insight into the underlying biological phenomena affected by constitutive *Avr2* expression, two complementary methods were applied. On the one hand, a scoring-based resampling method was applied to identify significantly overrepresented Gene Ontology (GO) classes (Lee et al., 2005). GO terms provide three structurally controlled vocabularies (ontologies) to describe genes and gene products in terms of their associated biological processes, their associated cellular components, and their molecular and biochemical functions in a species-independent manner (Ashburner et al., 2000; Harris et al., 2004). As input, all *t* test P values from the probe set comparisons across the respective conditions were used. To perform this method, the ErmineJ (Lee et al., 2005) software was used. This analysis revealed that genes participating in regulation of actin cytoskeleton reorganization, photosynthesis, and biosynthesis of nitrogenous compounds, such as amino acids and glucosinolates, were differentially regulated, in addition to pathways related to wounding, oxidative stress, and jasmonic acid/ethylene/salicylic acid signaling (see Supplemental Data Set 1 online). The cellular compartments associated with the differentially regulated gene products are involved in the secretory pathway and the exterior of the cell (apoplast), in addition to components that play a role in protein phosphorylation, reactive oxygen stress, and proteasome function (see Supplemental Data Set 1 online). This is also reflected by the molecular function of the products of the differentially regulated genes (see Supplemental Data Set 1 online).

To further characterize the transcriptional response of *At-Avr2-A* plants, we employed Gene Set Enrichment Analysis (GSEA), which places gene products in a broader context covering biochemical, metabolic, and signaling networks (Subramanian et al., 2005). This method is widely used to analyze human and murine transcriptome data (van Baarlen et al., 2008). To perform GSEA for *Arabidopsis*, a database was constructed through transforming *Arabidopsis* Kyoto Encyclopedia of Genes and Genomes (KEGG; http://www.genome.jp/kegg/pathway.html) pathway information that represents current knowledge on molecular and biochemical networks. Furthermore, the database was supplemented with various expressed gene sets reported in literature. The resulting database was queried with the set of 880 Avr2-triggered differentially expressed *Arabidopsis* genes (Table 1). This showed that the obtained gene set was enriched for genes that are similarly found in *Arabidopsis* challenged with *P. syringae* or *Escherichia coli*, treated with bacterial effectors, or treated with pathogen phytotoxins such as *P. syringae* coronatine, *Alternaria alternata* AAL toxin, and *Fusarium oxysporum* Nep1 toxin (Table 1). Moreover, genes involved in the host secretory pathway were also overrepresented (Table 1). Overall, it can be concluded from the global transcriptional profiling that *Avr2* expression in *Arabidopsis* triggers a global transcription pattern that reflects pathogen challenge, suggesting that basal defense is affected rather than common physiological processes.

Identification of *Arabidopsis* Cys Proteases Targeted by Avr2

It has previously been demonstrated that Avr2 binds to, and inhibits, the tomato apoplastic Cys protease Rcr3 (Rooney et al., 2005). To investigate whether Avr2 also inhibits *Arabidopsis* Cys proteases, we applied protease activity profiling (van der Hoorn et al., 2004) on wild-type and *At-Avr2-A* plants. Total protein extracts from unchallenged soil-grown plants were treated with DCG-04, a biotinylated derivative of the irreversible Cys protease inhibitor E-64 that reacts with the catalytic Cys residue in an activity-dependent manner (Greenbaum et al., 2002) to biotinylate active Cys proteases (van der Hoorn et al., 2004; Rooney et al., 2005). Subsequently, the biotinylated Cys proteases were detected on protein gel blots using streptavidin-coupled horseradish peroxidase (HRP), showing two major bands of biotinylated Cys proteases in wild-type Col-0 plants migrating around 25 kD and around 30 kD. These bands can be fully competed by pretreatment with an excess of E-64 prior to labeling, indicating that DCG-04 specifically binds to Cys proteases (Figure 2). Interestingly, treatment of total protein extracts from unchallenged soil-grown Col-0 plants with an excess of Avr2 likewise prevented subsequent labeling by DCG-04, demonstrating that Avr2 is also able to inhibit Cys proteases in wild-type *Arabidopsis* plants (Figure 2). When compared with the progenitor Col-0 plants, a slightly different pattern of active Cys proteases was observed in protein gel blots of *Avr2*-expressing *At-Avr2-A* plants. In contrast with Col-0 plants, in *At-Avr2-A* plants, the 30-kD band was more intense than the 25-kD band. Pretreatment of the extracts

Table 1. GSEA of the Transcriptome of 4-Week-Old Unchallenged *Avr2*-Expressing *Arabidopsis* Plants

Description of Gene Set	Size[a]	Tag[b]	P Value[c]	FDR Q Value[d]	Reference
Type III–induced genes of *P. syringae* coronatine mutant	264	38%	0.000	0.000	Thilmony et al. (2006); see Supplemental Table 5 online
P. syringae type III–induced genes	263	38%	0.000	0.000	Thilmony et al. (2006); see Supplemental Table 7 online
SNARE interactions in vesicular transport	46	63%	0.000	0.000	KEGG pathway
Ndr1-specific upon challenge with *P. syringae* expressing *AvrRpt2*	50	42%	0.000	0.001	Sato et al. (2007)
Alternaria (AAL) toxin-induced programmed cell death at 72 h	92	25%	0.000	0.001	Gechev et al. (2004)
N-glycan biosynthesis	20	60%	0.003	0.003	KEGG pathway
Alternaria (AAL) toxin-induced programmed cell death at 48 h	92	24%	0.000	0.005	Gechev et al. (2004)
Alternaria (AAL) toxin-induced programmed cell death at 24 h	87	21%	0.003	0.024	Gechev et al. (2004)
Nucleotide sugars metabolism	15	20%	0.029	0.047	KEGG pathway
P. syringae coronatine-regulated genes	323	28%	0.000	0.053	Thilmony et al. (2006); see Supplemental Table 6 online
Salicylic acid–repressed auxin signaling pathway	20	45%	0.036	0.075	Wang et al. (2007)
Glycan biosynthesis	19	47%	0.056	0.142	KEGG pathway
Ribosome	180	59%	0.008	0.134	KEGG pathway
PAMP-repressed genes	115	28%	0.019	0.137	Thilmony et al. (2006); see Supplemental Table 2 online
Proteasome	40	40%	0.101	0.223	KEGG pathway
E. coli strain O157:H7-induced genes	220	33%	0.042	0.253	Thilmony et al. (2006)
Metabolism of xenobiotics by cytochrome p450	17	47%	0.157	0.270	KEGG pathway
Alternaria (AAL) toxin-induced programmed cell death at 7 h	55	18%	0.110	0.260	Gechev et al. (2004)
F. oxysporum Nep1 toxin-induced death	432	15%	0.047	0.323	Bae et al. (2006)
P. syringae–regulated genes	154	27%	0.129	0.369	Thilmony et al. (2006); see Supplemental Table 2 online

[a]Number of genes present in the gene set or KEGG pathway after filtering out those genes not in the expression data set.
[b]The percentage of gene hits before or after the peak in the running enrichment score gives an indication of the percentage of genes contributing to the enrichment score.
[c]Nominal P value, the statistical significance of the enrichment score.
[d]False discovery rate Q value.

with an excess of E-64 resulted in absence of active Cys proteases, since no biotinylated signals were obtained on the blot (Figure 2), confirming that the signals were derived from active Cys proteases. Interestingly, treatment of the sample with an excess of Avr2 did not prevent subsequent labeling by DCG-04 (Figure 2), demonstrating that the composition of the set of active Cys proteases in *At-Avr2-A* plants is different from that in Col-0 plants and contains proteases that cannot be inhibited by Avr2.

To identify the Cys proteases present in the extracts, biotinylated proteins present in DCG-04–labeled protein extracts were purified on streptavidin magnetic beads and subjected to liquid chromatography–tandem mass spectrometry (LC-MS/MS) analysis. In the progenitor Col-0 *Arabidopsis* plants, seven Cys proteases could be identified, including aleurain, aleurain-like, cathepsin B, CPR1, RD21a, XCP1, and XCP2 proteases (Table 2). In the extract that was treated with an excess of E-64 prior to DCG-04 labeling, no proteases were identified at all. However, treatment of the Col-0 extract with an excess of Avr2 resulted in the detection of only cathepsin B (Table 2). This demonstrates that Avr2 is able to inhibit aleurain, aleurain-like, CPR1, RD21a, XCP1, and XCP2 because it can compete with DCG-04 for binding to the protease.

In *At-Avr2-A* plants, four Cys proteases were detected, aleurain, aleurain-like, RD21a, and cathepsin B, while CPR1, XCP1, and XCP2 were not detected (Table 2), demonstrating that constitutive *Avr2* expression inactivates or represses CPR1, XCP1, and XCP2. Treatment of the extract of *At-Avr2-A* plants with an excess of E-64 prior to DCG-04 labeling resulted in the absence of detectable aleurain, aleurain-like, RD21a, and cathepsin B. However, treatment of the extract with an excess of Avr2 did not result in the absence of detectable aleurain, aleurain-like, RD21a, and cathepsin B, although the number of peptides derived from RD21A was significantly reduced. This suggests that the affinity of Avr2 for aleurain, aleurain-like, and RD21a is lower than for CPR1, XCP1, and XCP2.

Overall, from the Cys profiling experiments in *Arabidopsis* it can be concluded that the Cys proteases in *Arabidopsis* can be divided into three classes based on their interaction with Avr2: proteases that cannot be inhibited by Avr2 (cathepsin B),

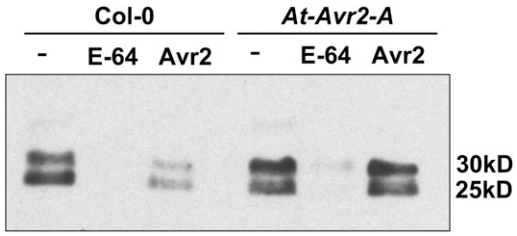

Figure 2. Avr2 Inhibits Cys Proteases in *Arabidopsis*.

Protein gel blot of total protein extracts from *Arabidopsis* transformants expressing *C. fulvum Avr2* (*At-Avr2-A*) and corresponding progenitor Col-0 plants upon treatment with the biotinylated Cys protease inhibitor DCG-04 and isolation using streptavidin-coated beads. Active Cys proteases are detected with streptavidin-coupled HRP. Prior to labeling with DCG-04, the extracts received no treatment (−) or were treated with either E-64 or Avr2.

proteases for which Avr2 has clear affinity (CPR1, XCP1, and XCP2), and proteases for which Avr2 has some (based on incidental treatment of Col-0 extracts) but rather low (based on the constitutive Avr2 presence in *At-Avr2* plants) affinity (aleurain, aleurain-like, and RD21a).

Production and Characterization of *Avr2* Transgenic Tomato Lines

Using *Agrobacterium tumefaciens*–mediated transformation, transgenic MoneyMaker-*Cf-0* tomato plants (MM-*Cf-0*) were generated for constitutive expression of *C. fulvum Avr2*. Two independent lines with a single-copy insert of the transgene were retained for further analysis (MM-*Avr2-A* and MM-*Avr2-B*; collectively, MM-*Avr2* lines). Similar to *Arabidopsis*, no macroscopically visible phenotypic anomalies were observed in these lines when grown under standard greenhouse conditions (see Supplemental Figure 3A online). It has previously been shown that tomato seeds expressing the *Cf-4* or *Cf-9* resistance gene in combination with the cognate *Avr* gene readily germinate but develop a systemic HR within a few days after emergence of the hypocotyls and die (Hammond-Kosack et al., 1994; Cai et al., 2001; Stulemeijer et al., 2007). Similarly, a cross between MM-*Avr2-A* and MM-*Cf-2* resulted in viable seeds that germinated at room temperature, but eventually all *Cf-2* × *Avr2* seedlings died, whereas seedlings from both parental lines retained normal germination and growth characteristics (see Supplemental Figure 3C online). In addition, apoplastic fluids from MM-*Avr2* plants, but not those from the progenitor MM-*Cf-0* line, resulted in a clearly visible HR 4 d after injection into leaves of MM-*Cf-2* plants, confirming the presence of biologically active Avr2 in the apoplast of MM-*Avr2* lines (see Supplemental Figure 3B online).

Heterologous Expression of *Avr2* in Tomato Promotes *C. fulvum* Colonization

Using the MM-*Avr2* lines, we determined whether *Avr2* expression enhances the virulence of a wild-type strain of *C. fulvum* lacking functional Avr2. Four-week-old MM-*Avr2-A* plants and control MM-*Cf-0* plants were inoculated with conidia of a race 2 *C. fulvum* strain that lacks functional Avr2, and disease progression was monitored up to 3 weeks after inoculation. Visual inspection showed that *Avr2*-expressing plants were clearly more susceptible to this strain as colonization occurred faster than on MM-*Cf-0* plants (Figure 3). At 14 d after inoculation (DAI), conidiophores of *C. fulvum* emerged from the *Avr2*-expressing plants, while conidiophores were not yet observed on MM-*Cf-0* leaves (Figure 3A). We have previously shown that *C. fulvum* biomass can be measured by real-time PCR determination of *C. fulvum* actin levels (van Esse et al., 2007; Bolton et al., 2008). The enhanced colonization of MM-*Avr2* plants was confirmed by real-time PCR at 10 and 14 DAI, showing that MM-*Avr2* plants

Table 2. Active Cys Proteases Identified in Total Extracts of *Arabidopsis* Plants

Probe		DCG-04 (Biotinylated E-64)					
Treatment		Noninoculated					
Plant genotype		Col-0			*At-Avr2-A*		
Competitor		No Competitor[a]	Excess E-64	Excess Avr2	No Competitor	Excess E-64	Excess Avr2
Protease	XCP1 (O65493)[b]	**4** (2-0-1-1-0)	–	–	–	–	
	XCP2 (Q9LM66)	**11** (6-3-1-1-0)	–	–	–	–	
	Cathepsin B (Q9ZSI0)[c]	**12** (10-1-1-0-0)	–	**10** (6-1-1-1-1)	**10** (10-0-0-0-0)	–	**11** (11-0-0-0-0)
	RD21A (P43297)	**13** (12-1-0-0-0)	–	–	**10** (9-1-0-0-0)	–	**3** (3-0-0-0-0)
	CPR1 (Q9LT77)[d]	**5** (2-1-1-1-0)	–	–	–	–	
	Aleurain (Q8H166)	**9** (7-2-0-0-0)	–	–	**21** (18-1-0-1-1)	–	**21** (19-0-0-1-1)
	Aleurain-like (Q8RWQ9)	**7** (5-1-0-0-1)	–	–	**4** (4-0-0-0-0)	–	**8** (7-1-0-0-0)

[a] Numbers in bold correspond to the amount of peptides observed that belong to the corresponding Cys protease. Numbers in parentheses show the position of the peptide in the cross-correlation scan list (from first through fifth rank, respectively). The filtering criteria were as described by Peng et al. (2003).

[b] Codes in parentheses are TrEMBL entry codes.

[c] The same peptides were identified for Cathepsin B (Q94K85).

[d] The same peptides were identified for pseudotzain (Q3EB42).

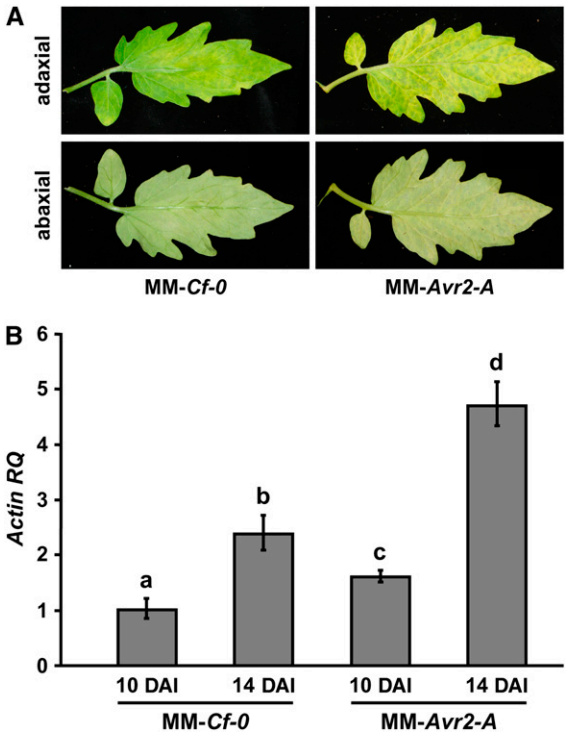

Figure 3. *Avr2*-Expressing Tomato Is More Susceptible to Race 2 *C. fulvum*.

(A) Typical disease symptoms on the adaxial and abaxial sides of the leaflets after inoculation with a race 2 *C. fulvum* strain of *Avr2*-expressing tomato (MM-*Avr2-A*) compared with those of the progenitor line (MM-*Cf-0*) at 11 DAI. The adaxial side of MM-*Avr2-A* shows enhanced *C. fulvum*–induced chlorosis compared with MM-*Cf-0*. On the abaxial side, a pale yellowish shade around the main veins indicative of fungal growth is observed on MM-*Avr2-A* and not on MM-*Cf-0*.

(B) Quantitative real-time PCR of fungal colonization levels (*Actin RQ*) determined by comparing *C. fulvum* actin transcript levels (a measure for fungal biomass) relative to tomato actin transcript levels (for equilibration) on MM-*Avr2-A* plants compared with the progenitor line (MM-*Cf-0*) at 10 and 14 DAI. The MM-*Cf-0* at 10 DAI is set to 1. Bars represent the average and SD of a minimum of three measurements, and different letters above the data points represent significant ($P < 0.05$) differences between means.

accumulated more *C. fulvum* biomass faster than MM-*Cf-0* plants (Figure 3B). Similarly, enhanced colonization of MM-*Avr2-B* plants was observed when compared with control MM-*Cf-0* plants upon inoculation with race 2 *C. fulvum* (see Supplemental Figure 4A online). Experiments with another natural *C. fulvum* strain that lacked a functional *Avr2* gene provided similar results (see Supplemental Figure 4B online), strongly suggesting that Avr2 is a virulence factor of *C. fulvum*.

Silencing of *Avr2* in *C. fulvum* Compromises Virulence on Tomato

Gene silencing has been used to reduce the expression of *C. fulvum* effector genes (van Esse et al., 2007; Bolton et al., 2008). To determine the role of Avr2, gene silencing was performed in a race 5 strain of *C. fulvum* (that contains the wild-type *Avr2* gene) using an inverted-repeat fragment of the *Avr2* gene driven by the constitutive *ToxA* promoter of the cereal pathogenic fungus *Pyrenophora tritici-repentis* (Ciuffetti et al., 1997). Several putative *Avr2* inverted-repeat (*Avr2*-IR) transformants were obtained, three of which were used for further analysis. Growth of these transformants in vitro on potato dextrose agar was indistinguishable from that of the progenitor strain (see Supplemental Figure 5 online). Since *C. fulvum* effector genes show no or low and variable expression when cultured in vitro (Thomma et al., 2006), 4-week-old MM-*Cf-0* tomato plants were inoculated with the three independent *Avr2*-IR transformants to determine whether the introduction of the *Avr2*-IR resulted in silencing. The in planta expression levels of *Avr2* were determined relative to the constitutively expressed *C. fulvum* actin gene to calibrate for the amount of fungal biomass in the sample at 14 DAI using real-time RT-PCR, showing a 60 to 70% reduction of *Avr2* expression in each of the transformants when compared with the progenitor *C. fulvum* strain (Figure 4A). Nevertheless, these levels were still sufficient to trigger *Cf-2*–mediated resistance in MM-*Cf-2* plants since the *Avr2*-IR transformants were still avirulent, although the response to *Avr2*-IR transformants was less vigorous than to the progenitor *C. fulvum* strain (see Supplemental Figure 6 online). Virulence assays on MM-*Cf-0* tomato plants showed that the *Avr2*-IR transformants were substantially compromised in their ability to colonize tomato leaves when compared with the progenitor *C. fulvum* strain, as they progressed slower and sporulated later (Figure 4B). This reduction in biomass by the *Avr2*-IR transformants compared with the progenitor strain was confirmed by real-time PCR quantification of *C. fulvum* actin transcripts at 11 DAI (Figure 4C), demonstrating that Avr2 is a genuine virulence factor.

Heterologous Expression of *Avr2* in Tomato Enhances Disease Susceptibility

As *Avr2*-expressing *Arabidopsis* transgenic lines showed increased susceptibility toward various pathogens, we analyzed *Avr2*-expressing tomato lines for increased susceptibility toward *P. infestans*, *B. cinerea*, and *Verticillium dahliae*. For *P. infestans*, no difference in susceptibility was observed between the MM-*Avr2* lines and the progenitor MM-*Cf-0* line. However, significantly more necrosis developed upon *B. cinerea* inoculation on MM-*Avr2* lines than on the progenitor MM-*Cf-0* line (Figure 5A), which correlated with enhanced fungal colonization as observed by microscopy analysis (Figure 5B). While on MM-*Cf-0* plants germinating *B. cinerea* conidia formed only short hyphae, hyphae on MM-*Avr2* plants were significantly longer and grew into the plant tissue (Figure 5B; see Supplemental Figure 7 online). We subsequently tested the susceptibility of MM-*Avr2* plants toward the vascular pathogen *V. dahliae* (Fradin and Thomma, 2006). Also with this pathogen, enhanced disease development was observed on MM-*Avr2* plants compared with the progenitor MM-*Cf-0* line. In this case, enhanced fungal colonization is reflected by stronger stunting, considerably reduced stem diameter, and stronger wilting symptoms in *Avr2*-transgenic lines (Figure 5C).

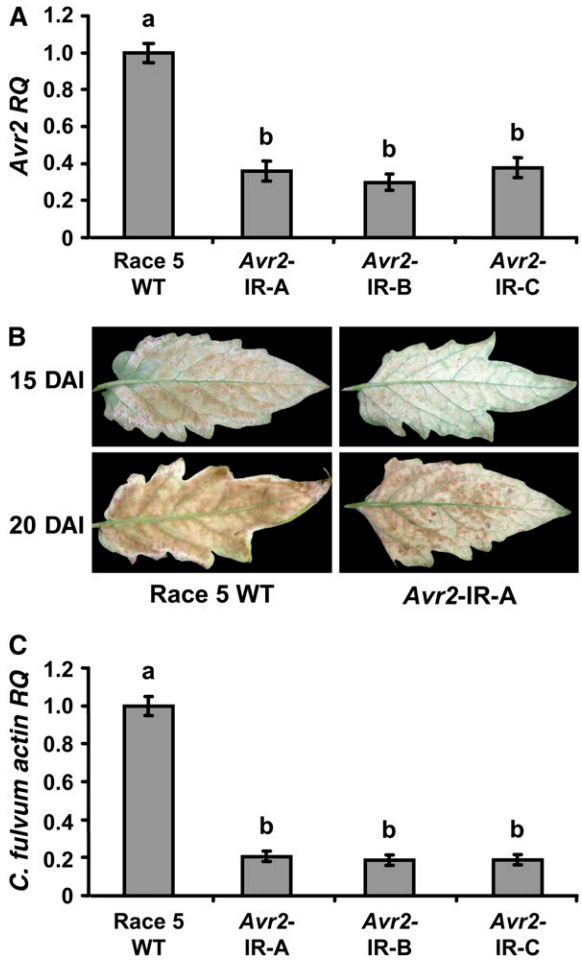

Figure 4. Silencing of *Avr2* in *C. fulvum* Decreases Virulence on Tomato.

(A) Quantitative real-time PCR of *Avr2* transcript levels (*Avr2 RQ*) of a virulent wild-type race 5 strain and three independent *Avr2*-silenced race 5 strains on MM-*Cf-0* tomato plants. *Avr2* transcript levels are shown in three independent *Avr2*-silenced *C. fulvum* transformants (*Avr2*-IR-A to -C) relative to *C. fulvum* actin transcript levels (for equilibration) compared with the progenitor strain (Race 5 WT, set to 1) at 11 DAI. Different letters above the data points represent significant ($P < 0.05$) differences between means.
(B) Typical disease symptoms after inoculation of MM-*Cf-0* tomato plants with the *Avr2*-silenced *C. fulvum* transformant *Avr2*-IR-A, as a representative example, compared with the progenitor strain (Race 5 WT), monitored at 15 and 20 DAI.
(C) Quantitative real-time PCR of fungal colonization (*C. fulvum actin RQ*) by comparing *C. fulvum* actin transcript levels (as a measure for fungal biomass) relative to tomato actin transcript levels (for equilibration) for three independent *Avr2*-silenced *C. fulvum* transformants (*Avr2*-IR-A to -C) compared with the progenitor strain (Race 5 WT, set to 1) at 11 DAI. Different letters above the data points represent significant ($P < 0.05$) differences between means.

In our laboratory, we have also established a successful soil-based *V. dahliae* infection assay for *Arabidopsis* (Fradin and Thomma, 2006). Like for tomato, we found that *At-Avr2* plants were more susceptible to *V. dahliae* than progenitor Col-0 plants (Figure 5D).

Identification of Tomato Cys Proteases Targeted by Avr2

Apoplastic fluid obtained from a time-course experiment of MM-*Cf-0* plants inoculated with a natural strain of *C. fulvum* lacking functional Avr2 (Boukema, 1981) was assessed for the presence of active Cys proteases with biotinylated E-64. A protein gel blot, using streptavidin coupled to HRP for detection, demonstrated that inoculation of tomato with *C. fulvum* results in the induction of several active apoplastic Cys proteases (Figure 6). Compared with 0 DAI, at 5 and 7 DAI, more bands appeared while the intensities of the bands also increased, resulting in three major signals of 25, 30, and 37 kD at 7 DAI. The observed signals could fully be competed with an excess of E-64 prior to labeling, while they were largely competed by pretreatment with Avr2 (Figure 6). This demonstrates that Avr2 is able to inactivate multiple Cys proteases in tomato, as was also observed for *Arabidopsis*.

To identify the different tomato proteases, a large-scale labeling and purification experiment was performed. Cys proteases present in apoplastic fluids of noninoculated MM-*Cf-0* plants were labeled with DCG-04, and biotinylated proteins were isolated using streptavidin beads and subsequently identified with LC-MS/MS. Seven active Cys proteases could be identified in the apoplast of noninoculated MM-*Cf-0* leaves, including Rcr3, Pip1, TDI65, aleurain, glycinain, and two cathepsin B proteases (Table 3). Upon inoculation of MM-*Cf-0* plants with a natural strain of *C. fulvum* lacking a functional *Avr2* gene, the same proteases were identified except glycinain, which disappeared upon infection (Table 3). This demonstrates that *C. fulvum* infection results in larger amounts of active Cys proteases that are already present in noninoculated tomato plants.

To determine potential targets of the Cys protease inhibitor Avr2, apoplastic fluids of noninoculated tomato leaves were treated with an excess Avr2 prior to labeling with DCG-04. In the extract that was treated with an excess of E-64 prior to DCG-04 labeling, no proteases were identified at all. However, after Avr2 treatment, the Rcr3, Pip1, and glycinain proteases were no longer biotinylated by DCG-04, while TDI65, aleurain, and two cathepsin B proteases were still detected, showing that Avr2 has the highest affinity for Rcr3, Pip1, and glycinain (Table 3). The DCG-04 assay actually assesses the ability of Avr2 to block the binding of DCG-04 to active Cys proteases. To directly assess the potential of Avr2 to interact with target Cys proteases rather than assessing its competitive ability to block DCG binding, Avr2 was labeled with biotin and used as bait to isolate and identify interacting apoplastic Cys proteases with streptavidin beads in apoplastic fluids of noninoculated tomato leaves. In this approach, the Rcr3, Pip1, TDI65, and aleurain proteases were found to bind to Avr2 (Table 3).

In addition to the noninoculated and *C. fulvum*–inoculated MM-*Cf-0* plants, we performed protease activity profiling on apoplastic fluids from noninoculated MM-*Avr2* plants. Compared with noninoculated MM-*Cf-0* plants, five of the seven active Cys proteases were also identified in MM-*Avr2* plants, the exceptions being Rcr3 and glycinain (Table 3). Interestingly, fewer peptides were identified for Pip1 and glycinain, suggesting that the relative amount of these proteases is reduced in the extract, which is also observed in extracts of MM-*Cf-0* plants upon pretreatment with Avr2 prior to DCG-04 labeling.

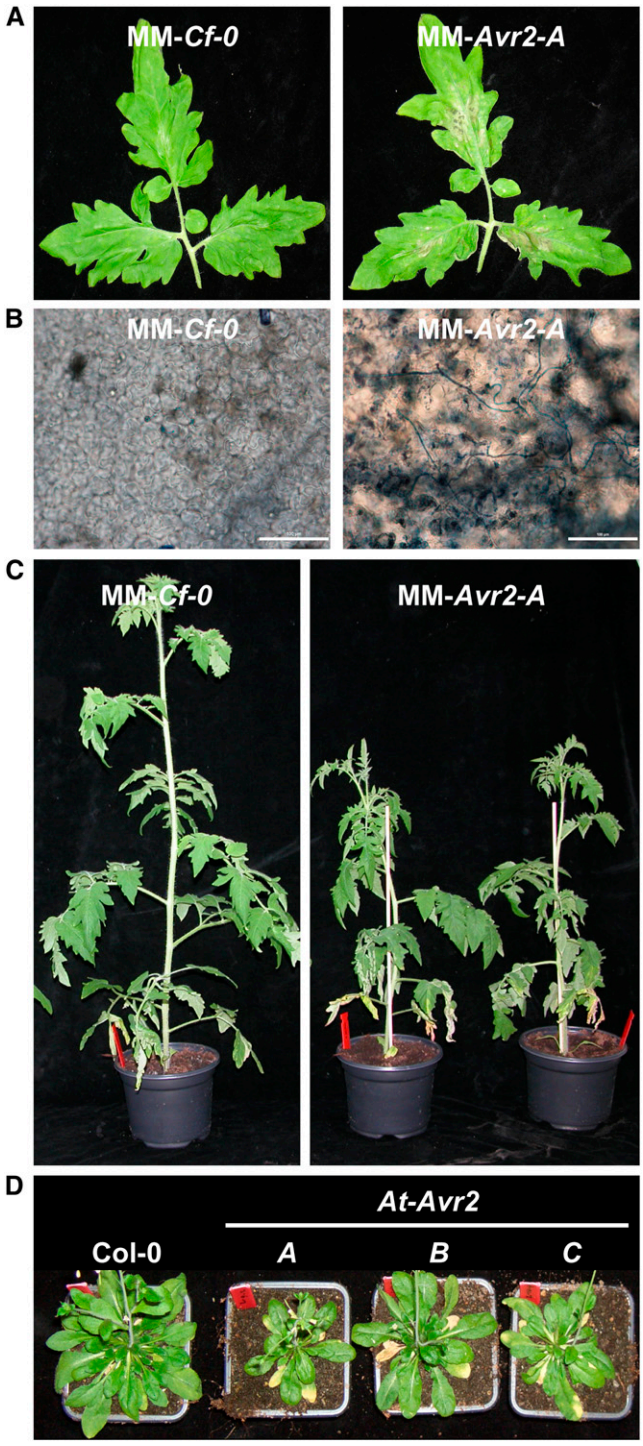

Figure 5. *Avr2*-Expressing Plants Are More Susceptible to *V. dahliae* and *B. cinerea*.

(A) Typical appearance of *Avr2*-expressing tomato leaves (MM-*Avr2-A*) compared with the progenitor line (MM-*Cf-0*) upon inoculation with *B. cinerea* at 3 DAI.

(B) Microscopy observation of *Avr2*-expressing cleared tomato leaves (MM-*Avr2-A*) compared with the progenitor line (MM-*Cf-0*) upon inoculation with *B. cinerea* at 2 DAI after staining of fungal hyphae and dead plant cells with Trypan blue.

(C) Typical appearance of *Avr2*-expressing tomato plants (MM-*Avr2-A*) compared with the progenitor line (MM-*Cf-0*) upon inoculation with *V. dahliae* at 2 weeks after inoculation.

(D) Typical stunting induced by *V. dahliae* on three independent *Avr2*-expressing *Arabidopsis* lines (*At-Avr2-A* to *-C*) compared with the progenitor line (Col-0) at 2 weeks after inoculation.

Overall, from the Cys profiling experiments in tomato it can be concluded that the tomato Cys proteases can be divided in three classes based on their interaction with Avr2: proteases that cannot be inhibited by Avr2 (cathepsin B), proteases for which Avr2 has clear affinity (Rcr3 and Pip1), and proteases for which Avr2 has lower affinity (TDI-65, aleurain, and glycinain). The inhibition of several extracellular host proteases by Avr2 is likely to cause the more susceptible phenotype. At present, biochemical evidence for the inhibition of protease activity by Avr2 only exists for Rcr3 (Rooney et al., 2005). However, *Cf-2/rcr3-3* mutants that lack Rcr3 due to a premature translational stop codon in the *Rcr3* gene (Krüger et al., 2002) did not show enhanced susceptibility toward race 2 *C. fulvum* strains that lack functional Avr2 when compared with *Cf-2/Rcr3* plants (see Supplemental Figure 8 online), suggesting that loss of Rcr3 function alone is not sufficient for the enhanced disease susceptibility. Therefore, it is likely that the simultaneous inhibition of several host proteases by Avr2 causes the observed enhanced disease susceptibility phenotypes in different pathosystems.

DISCUSSION

In resistant tomato plants, the protease inhibitory activity of *C. fulvum* Avr2 that results in modulation of the papain-like Cys protease Rcr3 is monitored by the Cf-2 protein, which results in *Cf-2*–mediated disease resistance (Rooney et al., 2005). Here, we show that Avr2 is a general Cys protease inhibitor that targets additional host proteases, which makes it a genuine virulence factor for *C. fulvum* that is also able to enhance the virulence of several other fungal plant pathogens on both tomato and *Arabidopsis*.

C. fulvum Avr2 Targets the Host Proteolytic Machinery

In this study, Cys protease activity profiling was performed using the biotinylated E-64 inhibitor of a C1 class of Cys proteases, DCG-04. The profiling assays in tomato and *Arabidopsis* resulted in the identification of several extracellular Cys proteases that interact with Avr2. Several proteases were identified in both hosts, cathepsin B and aleurain(-like), but also tomato TDI65, which is the homolog of *Arabidopsis* RD21A (Harrak et al., 2001). For the tomato proteases Rcr3 (Krüger et al., 2002) and Pip1 (Tian et al., 2007), no clear *Arabidopsis* homolog was identified. Likewise, for the *Arabidopsis* proteases XCP1, XCP2, (Zhao et al., 2000), and CPR1, no clear tomato homolog could be identified. However, XCP1 and XCP2 have been reported as xylem-specific C1 Cys proteases (Zhao et al., 2000; Funk et al., 2002), and it should be noted that the *Arabidopsis* protease

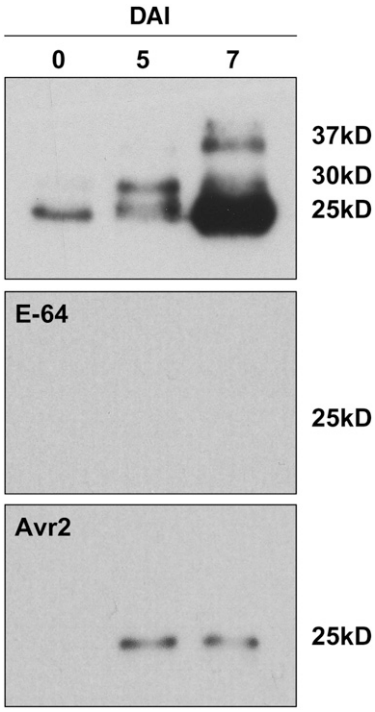

Figure 6. Active Cys Proteases Accumulate in the Tomato Apoplast Isolated from *C. fulvum*–Inoculated Leaves.

Protein gel blot of apoplastic fluids from tomato plants upon inoculation with *C. fulvum* at 0, 5, and 7 DAI upon treatment with the biotinylated Cys protease inhibitor DCG-04 and isolation using streptavidin-coated beads. Active Cys proteases were detected with streptavidin-coupled HRP. Prior to labeling with DCG-04, the extracts received no treatment (top panel) or were treated with E-64 (middle panel) or Avr2 (bottom panel).

activity profiling was performed on whole plant extracts, while the tomato profiling was performed on apopastic fluids. Thus, we are uncertain whether all identified *Arabidopsis* proteases indeed are present in the leaf apoplast.

To identify potential targets of Avr2 among the C1 proteases that irreversibly bind to E-64, the tomato and *Arabidopsis* extracts were treated with an excess of Avr2 prior to profiling with DCG-04. In both tomato and *Arabidopsis* extracts, Avr2 treatment prevented binding of DCG-04 to several proteases, including tomato Rcr3, Pip1, and glycinain, and all *Arabidopsis* proteases except the cathepsin B and cathepsin B–like proteases (Tables 2 and 3). The ability to prevent DCG-04 binding to these proteases demonstrates the ability of Avr2 to interact with these targets. To further characterize Avr2 targets in tomato, biotinylated Avr2 was used to fish for targets in apoplastic fluids. Using this strategy, Rcr3, Pip1, TDI65, and the aleurain protease were identified. It is conceivable that the kinetics of the interaction in which Avr2 is used to inactivate proteases and thus block DCG-04 binding may be different from the kinetics in the interaction in which Avr2 is used as a bait to fish for targets. It is currently not possible to predict whether the kinetics of the different approaches used here are biologically meaningful or not. Nevertheless, these results confirm that Avr2 has the potential to target multiple host Cys proteases of the C1 class and that so far no additional targets could be found in the tomato apoplast fluids.

The finding that Avr2 pretreatment did not prevent binding of DCG-04 to TDI65 and aleurain, although these proteases were identified when fishing with biotinylated Avr2, suggests that Avr2 interacts reversibly with these two proteases. Furthermore, it should be noted that due to the limitations of the current LC-MS/MS technology our assay detected only qualitative but not quantitative differences between samples, so even a large reduction in binding of DCG-04 to TDI65 and aleurain may remain unnoticed. Clearly, in both tomato and *Arabidopsis* extracts, Avr2 treatment did not prevent binding of DCG-04 to cathepsin B proteases, suggesting that Avr2 has no affinity for these proteases (Tables 2 and 3). Overall, our assays demonstrate that *C. fulvum* Avr2 targets several apoplastic papain-like Cys proteases of the host proteolytic machinery. Interestingly, our data furthermore show that, while *C. fulvum* Avr2 targets multiple host proteases, the tomato Cf-2 protein guards only Rcr3. This has also been observed for the *P. syringae* effectors AvrB, AvrRpm1, and AvrRpt2 that all target multiple host proteins of which only the basal defense regulator RIN4 is guarded by the cognate R proteins (Belkhadir et al., 2004; Lim and Kunkel, 2004; Chisholm et al., 2005). It was recently shown that treatment of tomato plants with the salicylic acid analog benzothiodiazole induces the accumulation of Pip1 and Rcr3 (Shabab et al., 2008). Similar to our observations, it was shown in a competition experiment that, after Avr2-treatment, the Rcr3 and Pip1 proteases were no longer biotinylated by DCG-04, while the aleurain and cathepsin B proteases were still detected, thus confirming our finding that Avr2 has multiple targets in tomato (Shabab et al., 2008).

Host Proteases Are Essential for Basal Defense

Pathogens and their hosts use proteolytic machineries to modulate the outcome of their interaction. On the one hand, several bacterial effectors have been identified that possess protease activity to degrade or modify host components (Hotson and Mudgett, 2004). For instance, *P. syringae* AvrPphB targets the host protein kinase PBS1 (Shao et al., 2003), and AvrRpt2 cleaves the *Arabidopsis* basal defense regulator RIN4 (Kim et al., 2005a, 2005b). Consistent with the guard hypothesis, in both cases the plant has developed guards to monitor this effector-mediated degradation (RPS5 and RPS2, respectively) that subsequently activate effector-triggered immunity. On the other hand, host proteases are important for defense against pathogens (van der Hoorn, 2008). For example, the *Arabidopsis* aspartic protease CDR1 is proposed to mediate a peptide signal system involved in the activation of inducible resistance against *P. syringae* (Xia et al., 2004), while the vacuolar Cys protease RD19 is required for RRS1-R resistance that is triggered by the PopP2 effector of *Ralstonia solanacearum* (M. Bernoux, A. Jauneau, T. Timmers, C. Brières, P.J.G.M. de Wit, Y. Marco, and L. Deslandes, unpublished data). Both RD19 and RRS1-R are targeted by PopP2 and are translocated to the nucleus where effector-triggered immunity is activated. Furthermore, several plant proteases have been implicated in the HR (D'Silva et al.,

Table 3. Active Cys Proteases Identified in Total Extracts of Tomato Plants

Probe		DCG-04 (Biotinylated E-64)							Biotinylated Avr2	
Treatment		Noninoculated					*C. fulvum* Race 2		Noninoculated	
Plant genotype		MM-*Cf-0*			MM-*Avr2-A*		MM-*Cf-0*		MM-*Cf-0*	
Competitor		No Competitor[a]	Excess E-64	Excess Avr2	No Competitor	Excess E-64	No Competitor	Excess E-64	No Competitor	Excess E-64
Protease	Rcr3 (TC128871)[b]	**5** (4-1-0-0-0)	–	–	–	–	**13** (8-1-1-2-1)	–	**7** (6-1-0-0-0)	–
	Pip1 (TC118154)	**17** (17-0-0-0-0)	–	–	**7** (6-1-0-0-0)	–	**53** (49-2-1-1-0)	–	**7** (7-0-0-0-0)	–
	Cathepsin B (TC162008)	**4** (4-0-0-0-0)	–	**3** (3-0-0-0-0)	**6** (6-0-0-0-0)	–	**3** (3-0-0-0-0)	–	–	–
	Cathepsin B (TC162009)	**9** (9-0-0-0-0)	–	**7** (7-0-0-0-0)	**11** (11-0-0-0-0)	–	**4** (4-0-0-0-0)	–	–	–
	TDI65 (TC124125)	**15** (15-0-0-0-0)	–	**7** (6-0-1-0-0)	**18** (17-1-0-0-0)	–	**18** (12-3-3-0-0)	–	**3** (3-0-0-0-0)	–
	Aleurain (TC116458)	**11** (11-0-0-0-0)	–	**9** (9-0-0-0-0)	**12** (12-0-0-0-0)	–	**13** (10-2-0-1-0)	–	**2** (2-0-0-0-0)	–
	Glycinain (TC124017)	**3** (2-1-0-0-0)	–	–	–	–	–	–	–	–

[a] Numbers in bold correspond to the amount of peptides observed that belong to the corresponding Cys protease. Numbers in parentheses show the position of the peptide in the cross-correlation scan list (from first through fifth rank, respectively). The filtering criteria were as described by Peng et al. (2003).

[b] Codes in parentheses are tentative consensus sequences of The Institute for Genomic Research.

1998; Solomon et al., 1999; Chichkova et al., 2004; Coffeen and Wolpert, 2004; Rojo et al., 2004; Woltering, 2004; Gilroy et al., 2007). It is therefore not surprising that pathogens use protease inhibitors during infection to target host proteases. Several secreted effector proteins from the oomycete pathogen *P. infestans* have been identified that display protease inhibitory activity (Tian et al., 2004, 2005, 2007). The Kazal-like Ser protease inhibitor directly interacts with the extracellular subtilisin-like protease PR protein P69B (Tian et al., 2004), while the Cys protease inhibitor EPIC2 interacts with the Cys protease Pip1 (Tian et al., 2007). However, while diverse roles of plant proteases in disease signaling have been established, a role as genuine defense molecule has so far not been demonstrated.

In this study, apoplastic delivery of Avr2 in *Arabidopsis*, a nonhost for *C. fulvum*, and in tomato resulted in enhanced susceptibility toward several fungal pathogens. In addition to the biotroph *C. fulvum*, these include the necrotrophic pathogens *B. cinerea* and *P. cucumerina* and the vascular pathogen *V. dahliae*. However, no enhanced susceptibility toward the avirulent fungal pathogen *A. brassisicola* and the bacterial pathogen *P. syringae* was observed in *Avr2*-expressing *Arabidopsis*. Likewise, disease development by the haustorial pathogens *H. parasitica*, *P. brassicae*, and *P. infestans* remained unaltered. Together, these results demonstrate that Avr2 expression compromises basal defense against pathogens that may be designated as extracellularly growing (nonhaustorial) virulent fungi. This likely reflects that pathogens that do not use haustoria or mechanisms for host cytoplasmic delivery of effector proteins, such as type III secretion or RxLR host targeting motifs, are more sensitive to apoplastic defenses.

Compromising specific defense mechanisms by Avr2, rather than merely disturbing host physiology, is not only supported by the disease susceptibility toward specific pathogens, but is further substantiated by transcriptional profiling of *Avr2*-expressing *Arabidopsis* plants in the absence of pathogen challenge. GSEA and ErmineJ analyses (Lee et al., 2005; Subramanian et al., 2005) were used to characterize the transcriptional response of *Arabidopsis* upon *Avr2* expression as a typical plant response to pathogens or pathogen-derived components. Both types of analyses are unbiased because no gene selection step is used as all expressed genes are included, and a score is computed based on all genes in a particular GO term or gene set. Genes involved in the regulation of actin cytoskeleton reorganization and typical responses to wounding, oxidative stress, jasmonic acid, ethylene, and salicylic acid (see Supplemental Data Set 1 online) were overrepresented in the expression profiles. Furthermore, genes associated with the secretory pathway and the exterior of the cell (apoplast) were also overrepresented.

Identification of Intrinsic Roles of Filamentous Pathogen Effectors

A role for secreted effectors in pathogen virulence has been demonstrated for only a few filamentous pathogens. Three in planta–secreted *C. fulvum* proteins, Ecp1, Ecp2, and Ecp6, have been implicated in full virulence of the pathogen (Laugé et al., 1997; Bolton et al. 2008). Similarly, two avirulence proteins from the barley powdery mildew fungus *Blumeria graminis* f. sp *hordei* and the *SIX1* avirulence protein from *F. oxysporum* f. sp *lycopersici* were shown to increase fungal infectivity on the respective hosts

(Rep et al., 2005; Ridout et al., 2006). The secreted effector proteins ATR1 and ATR13 from the oomycete *Arabidopsis* pathogen *H. parasitica* were also shown to contribute to pathogen virulence when delivered to the host by *P. syringae* (Sohn et al., 2007). However, in all these cases, the mechanism by which these effectors contribute to virulence is not yet understood. In a recent study to investigate the mechanism of action of a microbial effector, it was demonstrated that the *C. fulvum* effector protein Avr4 is a counterdefense factor that protects fungal cell walls against hydrolysis by plant chitinases through chitin binding activity and thus contributes to fungal virulence (van Esse et al., 2007). We have now shown that the *C. fulvum* effector protein Avr2 contributes to fungal virulence by targeting host proteases that are crucial for basal defense since *Avr2*-expressing tomato is more susceptible toward natural race 2 *C. fulvum* strains and *Avr2* silencing in a race 5 strain of *C. fulvum* clearly affected fungal aggressiveness.

Importantly, our results demonstrate that heterologous expression of secreted pathogen effectors in planta may successfully be used to uncover the intrinsic biological functions of these molecules. Moreover, depending on the nature of the effector target, the plant species used may even be a nonhost of the pathogen from which the effector is derived. We have recently used heterologous expression in *Arabidopsis* and tomato to show that the *C. fulvum* effector Avr4 is a genuine virulence factor (van Esse et al., 2007), and in this study, we used a similar approach for *Avr2*. Several virulence targets of Avr2 were identified both in *Arabidopsis* and in tomato, while increased susceptibility toward some of the same pathogens was demonstrated. This not only suggests that basal defense responses in different plant species are highly conserved but also that effector targets of different pathogens with diverse hosts may be orthologs (van Baarlen et al., 2007).

METHODS

All experiments have been performed a minimum of three times yielding similar results.

Cultivation of Microorganisms and Plants

Cladosporium fulvum and *Verticillium dahliae* were cultured at room temperature on half-strength potato dextrose agar (Oxoid) supplemented with 7 g/L technical agar (Oxoid). *Botrytis cinerea* (Brouwer et al., 2003) and *Plectosphaerella cucumerina* (Thomma et al., 2000) were cultured at room temperature on malt extract agar (Oxoid). *Phytophthora brassicae* isolate CBS686.95 was grown on V8 juice (Campbell Soups) agar (Erwin and Ribeiro, 1996) in the dark at 18°C. *Pseudomonas syringae* pv *tomato* DC3000 was cultured on King's B medium containing 200 μg/mL rifampicin.

Cf2/rcr3-3 tomato was described previously (Krüger et al., 2002). All tomato plants were grown in soil under standard greenhouse conditions: 21°C/19°C during the 16-h-day/8-h-night period, 70% RH, and 100 W/m^2 supplemental light when the intensity dropped below 150 W/m^2. *Arabidopsis thaliana* plants were grown in soil under similar greenhouse conditions with 21°C/18°C during the 16-h-day/8-h-night, 60% RH, and 100 W/m^2 supplemental light when the intensity dropped below 150 W/m^2.

Plant Transformations

Transgenic *Arabidopsis* (*At-Avr2-A* to *-C*; collectively called *At-Avr2* lines) and tomato (*Solanum lycopersicum*; MM-*Avr2-A* and MM-*Avr2-B*; collectively called MM-*Avr2* lines) expressing *C. fulvum Avr2* were generated in this study. Transgenic *Arabidopsis* expressing Avr4 and Avr9 have been described previously (van Esse et al., 2007). For in planta production of *C. fulvum* effectors, the sequence encoding each of the mature proteins was amplified (see Supplemental Table 1 online) and ligated into the binary pGREEN vector that contained the cauliflower mosaic virus 35S promoter for constitutive expression (Hellens et al., 2000) in frame with the sequence encoding the tobacco (*Nicotiana tabacum*) PR1a signal peptide for apoplastic targeting. This vector was introduced into *Agrobacterium tumefaciens* strain GV3101 by electroporation, and transformants were selected on Luria-Bertani (LB) medium supplemented with 50 μg/mL kanamycin and 25 μg/mL rifampicin. Subsequently, *Arabidopsis* transformants were generated using the floral dip method (Clough and Bent, 1998). First-generation transformants were selected on 50 μM kanamycin and subsequently transferred to soil. Several independent homozygous single insertion lines were selected, and T3 and T4 lines were used for inoculations.

Tomato transformations were performed using a modified protocol of Cortina and Culiáñez-Macià (2004). Seeds of the tomato cultivar Money-Maker (MM-Cf-0) were surface-sterilized (by incubation for 1 min in 70% ethanol and 25 min in 10% commercial bleach and rinsing three times in sterilized water), sown on Murashige and Skoog (MS) agar supplemented with sucrose (30 g/L), incubated in the dark in a growth chamber at 25°C for 2 d, and subsequently exposed to light. After ~10 d, cotyledons were harvested, cut in two, and placed upside down in Petri dishes containing precultivation medium (MS agar supplemented with 30 g/L sucrose, 2 mg/L naphthylacetic acid, and 1 mg/L 6-benzaminopurine, pH 5.8), after which the explants were covered with sterile filter paper imbibed with 2 mL of cocultivation medium (MS medium supplemented with 30 g/L sucrose, 2 g/L caseine hydrolysate [Duchefa], 1 g/L 2,4-D, and 0.5 mg/L kinetine [dissolved in 1 M NaOH], pH 6.5) and incubated in the dark for 24 h. Transgenic *A. tumefaciens* carrying the construct of interest was grown in LB medium containing 200 μM acetosyringone to an OD_{600} of 0.6, and after harvesting, the bacteria were resuspended in 75 mL of LB medium. Subsequently, the explants were incubated in the bacterial suspension for 5 to 10 min, dried on sterile filter paper, plated on precultivation medium, and incubated in the dark for 2 d. The explants were then transferred to regeneration medium (MS agar supplemented with 10 g/L sucrose, 10 g/L glucose, 2 mg/L zeatin riboside, 0.4 mg/L thiamine-HCL, 0.02 mg/L indole-3-acetic acid (IAA), 200 mg/L timentin [ticarcilline: potassium clavulanate, 15:1], 100 mg/L kanamycin, and 200 mg/L vancomycin, pH 5.8), incubated in the dark for 5 d, and transferred into light. The explants were transferred to fresh regeneration medium every 2 weeks. When calli appeared, they were transferred to shoot-inducing medium (MS agar supplemented with 10 g/L sucrose, 10 g/L glucose, 1 mg/L zeatin riboside, 0.02 mg/L IAA, 200 mg/L timentin, 100 mg/L kanamycin, and 200 mg/L vancomycin, pH 5.8). Upon meristem development, the explants were transferred to root-inducing medium (MS agar supplemented with 10 g/L sucrose, 10 g/L glucose, 0.02 mg/L IAA, 200 mg/L timentin, and 50 mg/L kanamycin, pH 5.8). Once roots developed, the plantlets were planted in soil and transferred to the greenhouse where they were grown under standard greenhouse conditions.

To verify apoplastic delivery of Avr2 in transgenic *Arabidopsis* and tomato plants, apoplastic fluid was isolated and injected into *Cf-2* tomato to obtain an HR according to de Wit and Spikman (1982).

Plant Inoculations

Inoculation of tomato with *C. fulvum* was performed as previously described (de Wit, 1977). To assess susceptibility of the *Avr2*-expressing

tomato lines, the Avr2-deficient *C. fulvum* strains 2.4.5 (Boukema, 1981) and 2.5.9 (Laterrot, 1986) were used. Briefly, 5-week-old soil-grown tomato plants were inoculated by spraying 5 mL of conidial suspension (10^6 conidia/mLl) onto the lower surface of the leaves. Subsequently, plants were kept at 100% RH for 48 h under a transparent plastic cover after which they we incubated at standard greenhouse conditions of a 16-h/8-h light/dark regime and 70% RH. Disease progression was monitored until 20 DAI.

Inoculation of tomato with *B. cinerea* (Brouwer et al., 2003) was performed as previously described (Díaz et al., 2002) with slight modifications. Briefly, a suspension of 10^6 conidia/mL in Gamborg's B5 medium (Duchefa Biochemie) supplemented with 10 mM Glc and 10 mM potassium phosphate, pH 6, was preincubated without shaking for 2 to 3 h at room temperature. Subsequently, 5-week-old soil-grown tomato plants were inoculated by spraying 5 mL of the inoculum onto the lower surface of the leaves. Plants were kept at 100% RH for 48 h under a transparent plastic cover after which they we incubated at standard greenhouse conditions. For microscopy analysis, *B. cinerea*–inoculated leaves were stained with lactophenol-trypan blue (Keogh et al., 1980) to visualize hyphal structures and dead plant cells. Leaves were destained and mounted in a chloral hydrate solution (2.5 mg/mL). Microscopy was preformed with a Nikon 90i epifluorescence microscope equipped with a Nikon DS-5Mc digital imaging system and Nikon NIS-elements AR software 2.3 (Nikon Instruments).

Phytophthora infestans inoculations on tomato were performed on detached leaves as described for potato (*Solanum tuberosum*; Vleeshouwers et al., 1999).

For inoculation with *V. dahliae*, 2-week-old soil-grown tomato plants were uprooted and inoculated by dipping the roots for 2 min in a conidial suspension (10^6 conidia/mL) in water. After replanting in soil, plants were incubated at standard greenhouse conditions of a 16-h/8-h light/dark regime and 70% RH. Disease progression was monitored until 20 DAI.

Inoculation of *Arabidopsis* plants with *B. cinerea*, *P. cucumerina*, *P. brassicae*, and *P. syringae* was performed on 4-week-old soil-grown plants. For *B. cinerea*, plants were inoculated by placing two 4-μL drops of a conidial suspension (5×10^5 conidia/mL) in 12 g/L potato dextrose broth (Difco) on each leaf. Inoculation with *P. cucumerina* was performed similarly, using an aqueous suspension containing 5×10^5 conidia/mL. For all pathogens, plants were incubated at 20°C, 100% RH, and a 16-h/8-h light/dark regime. Disease progression was scored at regular intervals, and representative pictures were taken at 4 DAI. Inoculation with *P. brassicae* was performed by placing 5-mm-diameter plugs of a 2-week-old *P. brassicae* agar plate culture onto *Arabidopsis* leaves. Subsequently, the plants were incubated at 16°C, 100% RH, and a 16-h/8-h light/dark regime. Inoculation with *P. syringae* pv *tomato* DC3000 was performed by spray inoculation of a bacterial suspension of 5×10^8 cfu/mL in 10 mM $MgCl_2$ and 0.05% Silwet L-77 (Lehle Seeds) onto the leaves until droplet runoff. Plants were incubated at 100% RH for 1 h, followed by incubation at 20°C, 60% RH, and a 16-h/8-h light/dark regime. Disease progression was scored at 4 DAI.

For inoculation of *Arabidopsis* with *V. dahliae*, 2-week-old soil-grown plants were uprooted and inoculated by dipping the roots for 2 min in a conidial suspension (10^6 conidia/mL) in water. After replanting in soil, plants were incubated at standard greenhouse conditions of a 16-h/8-h light/dark regime and 60% RH. Disease progression was monitored until 20 DAI.

Microarray Sample Preparation and Data Analyses

Samples used for microarray analyses were replicated three times in independent experiments, and each replication consisted of 10 *At-Avr2-A* plants and 10 Col-0 plants grown for 4 weeks under standard greenhouse conditions. In total, 30 *At-Avr2-A* plants and 30 Col-0 plants were assayed. All aboveground tissues were harvested, pooled, and flash-frozen in liquid nitrogen. For RNA extraction, the frozen leaves were ground using a spoon, and ~100 mg of the crushed material was homogenized in 1 mL Trizol reagent (Invitrogen). After addition of 200 μL chlorophorm and phase separation, isopropanol was added to the aqueous phase, which was subsequently further purified with the NucleoSpin RNA plant kit (Macherey-Nagel). In this way, total RNA was obtained that was hybridized onto six individual ATH1 Affymetrix *Arabidopsis* whole-genome arrays from *At-Avr2-A* and Col-0 plants grown in three independent replications. Probe preparations and GeneChip hybridizations were performed at ServiceXS (Leiden, The Netherlands).

Bioconductor packages (www.bioconductor.org; Gentleman et al., 2004) were used for analyzing the scanned Affymetrix arrays. The Bioconductor packages were integrated in the automated online MADMAX pipeline (https://madmax.bioinformatics.nl). The arrays were normalized using quantile normalization, and expression estimates were compiled using Rate Monotonic Analysis applying the empirical Bayes approach (Wu et al., 2004). They were considered of sufficiently high quality if they showed <10% of specks in fitPLM model images, were not deviating in RNA degradation and density plots, were not significantly deviating in NUSE and RLE plots, and were within each other's range in box plots. Differentially expressed probe sets were identified using linear models, applying moderated t-statistics that implement empirical Bayes regularization of standard errors (Smyth, 2004). P values were corrected for multiple testing using a false discovery rate (FDR) method (Storey and Tibshirani, 2003) that estimates type 1 (false positive) errors. For plants, an FDR < 0.05 cutoff is a suitable global value when arrays are of high quality (DeCook et al., 2006). Two complementary methods were applied to relate changes in gene expression to functional changes. One method is based on overrepresentation of GO terms (Lee et al., 2005). Another approach, GSEA, takes into account the broader context in which gene products function, namely, in physically interacting networks, such as biochemical, metabolic, or signal transduction routes (Subramanian et al., 2005). Both methods are unbiased because no gene selection step is used. Moreover, since a score is computed based on all genes in a particular GO term or gene set, the signal-to-noise ratio is boosted, allowing the detection of transcriptional programs that are distributed across an entire set of interacting genes yet are subtle at the level of individual genes. The higher FDR values reflect the biological variation that consists of between-plant variation and variation resulting from multiple (three) independent plant rearing and harvesting dates.

Avr2 Inverted-Repeat Transformants of *C. fulvum*

A fragment of the *Avr2*-coding sequence was amplified using cDNA from a compatible interaction between *C. fulvum* and tomato as template using the primer sequences as shown in Supplemental Table 1 online. Construction of the binary vector containing an inverted-repeat fragment of the *Avr2* gene and *A. tumefaciens*–mediated transformation of a race 5 strain of *C. fulvum* was performed as described (Bolton et al., 2008).

Quantification of *Avr2* Expression Levels and *C. fulvum* Biomass

The *Avr2*-IR transformants and the progenitor race 5 *C. fulvum* strain were inoculated onto 5-week-old MM-*Cf-0* tomato plants as described above. In each of three independently repeated experiments, leaf material was harvested 0, 3, 7, 11, and 16 DAI, flash-frozen in liquid nitrogen, and stored at −80°C until used for RNA analysis. Leaf samples consisted of three leaflets obtained from the 2nd, 3rd, and 4th compound leaves of two tomato plants. Total RNA was isolated from infected leaf material using the RNeasy kit (Qiagen) according to the manufacturer's instructions. Total RNA was used for cDNA synthesis using an oligo(dT) primer (see Supplemental Table 1 online) and the SuperScript II reverse transcriptase kit (Invitrogen) according to the manufacturer's instructions. Quantitative real-time PCR was conducted with primers given in Supplemental Table 1 online and using an ABI7300 PCR machine (Applied Biosystems) in

combination with the qPCR Core kit for SYBR Green I (Eurogentec). Real-time PCR conditions were as follows: an initial 95°C denaturation step for 2 min followed by denaturation for 15 s at 95°C and annealing/extension for 45 s at 60°C for 40 cycles and analyzed on the 7300 System SDS software (Applied Biosystems). To check for contamination with genomic DNA, real-time PCR was also performed on RNA without the addition of reverse transcriptase. Quantification of *C. fulvum* growth on MM-*Avr2* lines was performed similarly. Statistical analyses were performed in SPSS15.0 using one-way analysis of variance ($P < 0.05$) followed by the LSD and Dunnett t (two-sided) post hoc multiple comparisons.

Identification of Plant Cys Proteases Targeted by Avr2

Protein extracts from *Arabidopsis* and tomato were prepared and subjected to protease activity profiling with DCG-04 (van der Hoorn et al., 2004). In the profiling assays, the Cys protease inhibitors E-64 (110 μM final concentration) and His-FLAG-Avr2 (11 μM final concentration) were tested for their ability to compete with DCG-04 (220 nM final concentration) for binding to Cys proteases.

For tomato, apoplastic fluid was isolated from MM-*Cf-0* tomato inoculated with the Avr2-deficient *C. fulvum* strain (Boukema, 1981) at 14 DAI as previously described (van Esse et al., 2006), and 9 mL of fluid was used for protease activity profiling. To each extract, 1 mL of DCG-04 assay buffer (500 mM NaAc and 100 mM L-cysteine, pH 5.0) with DCG-04 (2.20 μM final concentration) was added and incubated at room temperature for 5 h. Subsequently, proteins were precipitated by addition of 20 mL of ice-cold acetone, washed with 70% (v/v) acetone, and subsequently dissolved in 1 mL TBS buffer (50 mM Tris/HCl and 150 mM NaCl, pH 7.5). The biotinylated Cys proteases were bound to magnetic streptavidin beads (Promega) by incubating for 16 h at 4°C. The beads were washed three times (50 mM Tris/HCl, 1.15 M NaCl, and 1% Triton X100) and subsequently rinsed twice with 50 mM NH_4HCO_3, pH 8.0. To reduce disulphide bridges, the beads were incubated with 50 mM DTT in 50 mM NH_4HCO_3, pH 8.0, for 2 h at 56°C, followed by alkylation of Cys residues by incubation in 50 mM iodoacetamide in 50 mM NH_4HCO_3, pH 8.0, for 2 h at 25°C in the dark. Finally, the immobilized Cys proteases were subjected to trypsin digestion. To this end, a fresh stock of 20 μg trypsin (Promega) in l00 μL 50 mM HAOc was prepared. Four microliters of this stock solution was diluted 10-fold in 100 mM NH_4HCO_3, pH 8.0, added to the beads, and incubated overnight at room temperature. Subsequently, another 4 μL of stock solution was added and incubated for 4 h at 37°C. The supernatant containing tryptic digests was separated from the magnetic beads, and 22 μL of the suspension was subjected to LC-MS/MS analysis.

For *Arabidopsis*, isolation of Cys proteases was performed as described previously (van der Hoorn et al., 2004), and reduction of disulphide bridges and tryptic digests was performed as described above for tomato.

To identify Cys proteases that directly bind to Avr2, the above-described protease activity profiling assays were performed in which DCG-04 was replaced by biotinylated Avr2 (67 μM final concentration). Biotinylated Avr2 was produced by labeling of *Pichia pastoris*–produced Avr2 (Rooney et al., 2005) using the No-Weigh Premeasured NHS-PEO4-Biotin microtubes (Pierce) according to the manufacturer's instructions.

The protein samples were analyzed with LC-MS/MS by injecting 18 or 20 μL of sample on a 0.10 × 32-mm Prontosil 300-3-C18H preconcentration column (Bischoff) at a flow of 3 or 6 μL/min for 10 min. Peptides were eluted from the preconcentration column onto a 0.10 × 200-mm Prontosil 300-3-C18H analytical column (Bischoff) with an acetonitril gradient at a flow of 0.5 μL/min. The gradient consisted of a 10 to 35% (v/v) acetonitril increase in water with 1 mL/L formic acid in 50 min. As a subsequent cleaning step, in 3 min the acetonitril concentration was increased to 80% (v/v) (with 20% water and 1 mL/L formic acid in both the acetonitril and the water).

Downstream of the analytical column, an electrospray potential of 1.8 kV was applied directly to the eluent via a solid 0.5-mm platina electrode fitted into a P875 Upchurch microT. Full scan positive mode MS spectra with three microscans (LCQ) or one microscan (LTQ) were measured between mass-to-charge ratios of 350 or 380 and 1400 on a LCQ classic or LTQ-Orbitrap (Thermo Fisher Scientific). The equipment was optimally tuned either by direct injection of 1 μM bradykinin or by injection of positive mode calibration mix at 0.5 μL/min via the electrospray device mentioned above. MS/MS scans of the three or four most abundant peaks in the MS scan were recorded in data-dependent mode.

All MS/MS spectra were analyzed with Bioworks 3.2 or 3.3.1 software (Thermo Fisher Scientific). A maximum of three differential modifications was set for oxidation of Met residues and deamidation of N and Q. Carboxamidomethylation of Cys residues was set as a fixed modification. An *Arabidopsis* database (downloaded from the European Bioinformatics Institute website at http://www.ebi.ac.uk/integr8/) to which the protein sequences BSA (P02769, bovine serum albumin precursor), Trypsin (P00760, bovine), Trypsin (P00761, porcine), Keratin K2C1 (P04264, human), and Keratin K1CI (P35527, human) were added was used for peptide identifications. The peptide identifications obtained were filtered in Bioworks with the following filter criteria: $\Delta Cn > 0.08$, Xcorr > 2 for charge state 1+, Xcorr > 1.5 for charge state 2+, Xcorr > 3.3 for charge state 3+, and Xcorr > 3.5 for charge state 4+ (Peng et al., 2003).

Accession Numbers

Sequence data from this article can be found in The Institute for Genomic Research or GenBank/EMBL databases under accession number AJ421629 (Avr2). Accession numbers for additional proteins are listed in Tables 2 and 3.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure 1. Characterization of *Avr2*-Expressing *Arabidopsis* Plants.

Supplemental Figure 2. *Avr2*-Expressing *Arabidopsis* Is More Susceptible to the Fungal Pathogen *B. cinerea*.

Supplemental Figure 3. Characterization of *Avr2*-Expressing Tomato Plants.

Supplemental Figure 4. *Avr2*-Expressing Tomato Is More Susceptible to Race 2 *C. fulvum*.

Supplemental Figure 5. Silencing of *Avr2* Expression in *C. fulvum* Does Not Compromise in Vitro Growth.

Supplemental Figure 6. Silencing of *Avr2* Expression in *C. fulvum* Compromises Cf-2–Mediated Immunity.

Supplemental Figure 7. *Avr2*-Expressing Plants Are More Susceptible to *B. cinerea*.

Supplemental Figure 8. *Cf-2* Tomato Lacking Rcr3 Is Not More Susceptible to Race 2 *C. fulvum*.

Supplemental Table 1. Primers Used in This Study.

Supplemental Data Set 1. ErmineJ Analysis of 4-Week-Old Unchallenged *Avr2*-Expressing *Arabidopsis* Plants.

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